REMARKS

Entry of the forgoing amendment is respectfully requested.

Point 4 of the office action set forth an objection to claim 10, based on a spelling error. Applicants have replaced claim 10 by claim 15 so that "Schizosaccharomyces" is presented in place of "Schizosoccharomyces." It is believed that this change overcomes the objection.

Claims 1-12 have been rejected under 35 USC 112, first paragraph, as failing to comply with the enablement requirement at point 5.

Applicants respectfully disagree with this rejection and provide the following comments. With respect to the rejected claims, the deposit of the C. albicans strains or of any microbial strains, is unnecessary. The strains are not required to practice the invention, since the use of the strains was for illustration only, and therefore the rejection should be withdrawn.

Additionally, claims 1-12 have been rejected under 35 USC 112, first paragraph, as failing to comply with the enablement requirement at point 6, and as failing to comply with the written description requirement at point 7.

Applicant respectfully disagrees, and provides the following comments. With respect to the rejected claims, the deposit of the C. albicans strains and the requested sequence listings are unnecessary. Again, the strains are not required to practice the invention, and their deposit, therefore, is not necessary. Further, the requested sequences are known or publicly available, thus their listing in the instant application is not required. A specification need not teach, and preferably leaves out, that which is well known in the art. Hybritech Inc. v. Monoclonal Antibodies Inc., 802 F. 2d 1367, 231 USPQ 81 (Fed. Cir. 1986).

Moreover, claims 1-12 have been rejected under 35 USC 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention at point 8.

With respect to the rejected claims, the "Csh3p molecule" is known or publicly available, and thus not an indefinite recitation. Also, claims 1, 5, 6, 8, 9 have been amended to recite "homolog instead of "analog." Homolog, as used in the specification (e.g., pg. 16, paragraph 80), refers to molecules that are structurally similar and have the same function as Csh3p. Thus, it is believed that this indefinite rejection has been overcome.

Further, claims 1-7 and 10-11 have been rejected under 35 USC 102(a) as being anticipated by Borisy, A.A. et al. 2003 (PNAS 100 (13): 7977-7892).

Applicants reduced their invention to practice prior to Borisy, as shown by the attached Declaration executed by the two co-inventors. As the invention was reduced to practice prior to the reference date, this rejection should be withdrawn.

In view of the foregoing, withdrawal of all rejections and allowance of this application are respectfully requested.

Respectfully submitted,

FULBRIGHT & JAWORSKI L.L.P.

By _____

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Attachment

LUD 5839 (10304693)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s)	:	Per O. LJUNGDAHL et al.
Serial No.	:	10/613,359
Filed	:	July 3, 2003
For	:	METHOD FOR MODULATING C. ALBICANS
Art Unit	:	1645
Examiner	:	Tammy K. Field

Commissioner of Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION

SIR:

THE UNDERSIGNED hereby declare as follows:

1. We are the properly named, co-inventors of the subject matter set forth in the above-referenced application.

2. We reduced the invention described in the above-referenced application to practice prior to June 24, 2003. We did the work described herein in Sweden, which is a member of the World Trade Organization. The United States is also a member of the World Trade Organization.

3. Attached hereto is a copy of a manuscript, submitted to the journal Cell prior to June 24, 2003. The manuscript evidences successful reduction to practice of the claimed invention prior to June 24, 2003.

4. We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

MAY 17, 2004
Date

Per O. LJUNGDAHL

May 17, 2004
Date

Paula MARTINEZ

Candida albicans Requires High-Capacity Amino Acid Uptake For Virulent Growth

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Running title: Amino Acid Uptake Affects C. albicans Virulence

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Summary

The C. albicans *CSH3* gene encodes a functional and structural homolog of Shr3p, a yeast protein that is specifically required for proper uptake and sensing of extracellular amino acids in S cerevisiae. A C. albicans *csh3Δ/csh3Δ* null mutant has a reduced capacity to take up amino acids, and is unable to switch morphologies on solid and in liquid media in response to inducing amino acids. *CSH3/csh3Δ* heterozygous strains display normal amino acid induced morphological switching, but cannot take up amino acids at wild-type rates. Strikingly both *CSH3/csh3Δ* heterozygous and *csh3Δ/csh3Δ* homozygous strains are unable to efficiently mount virulent infections in a mouse model. The haploinsufficiency phenotypes indicate that both *CSH3* alleles contribute to maintain high-capacity amino acid uptake in wild-type strains. These results demonstrate that C. albicans cells depend on amino acids, presumably due to their important role as nitrogen sources, to grow efficiently in mammalian hosts.

Introduction

Candida albicans is the most common human fungal pathogen causing a wide range of superficial mucosal diseases as well as life-threatening systemic infections in immunocompromised patients (Calderone and Fonzi, 2001; Corner and Magee, 1997; Odds, 1988; Odds et al., 2001). As is the case for all microorganisms, C. albicans must rely on its capacity to take up nutrients from the environment to survive and to proliferate. Little is known regarding what nutrients, e.g., the nitrogen sources, that are utilized by C. albicans during infectious growth within mammalian hosts. Nor is it well understood if nutrient availability affects the ability of this pathogen to circumvent host protective responses, including phagocytosis by macrophages and neutrophiles. It can be surmised that in order to survive within infected hosts, C. albicans cells must be able to rapidly adjust the efficacy of nutrient uptake systems in response to changes in the levels of nutrients present in the diverse microenvironments encountered during the infection process.

It is well recognized that apart from being metabolites for protein synthesis, amino acids have an important role in nitrogen homeostasis, and it has been shown that amino acids also act as morphogens that have the capacity to induce polymorphic fungi, such as C. albicans, to undergo morphological transitions (Dabrowa and Howard, 1981; Holmes and Shepherd, 1987; Land et al., 1975). The obligate diploid nature of C. albicans together with the lack of a known sexual phase and unusual codon usage has hampered the genetic manipulation of this organism. Consequently, limited information is available regarding how C. albicans cells take up amino acids, and it is not known whether C. albicans cells assess the availability of amino acids prior to or after their being taken up into cells. The well-studied yeast Saccharomyces cerevisiae possesses sophisticated systems to efficiently import amino acids from the external environment (for review see (Forsberg and Ljungdahl, 2001b). All of the known gene products involved in the positive regulatory circuits controlling the functional expression of amino acid permeases, the proteins that transport amino acids into cells, have homologs in the C. albicans genome (Table I).

Thus S. cerevisiae is likely to serve as a good model for understanding amino acid uptake in C. albicans.

Consistent with kinetic studies indicating the presence of multiple transport systems (Dabrowa and Howard, 1981; Kaur and Mishra, 1991; Prasad, 1987; Rao et al., 1986; Sychrova and Souciet, 1994), the C. albicans genome contains 22 ORFs that comprise a conserved gene family of amino acid permeases (AAPs) (Table 1). In S. cerevisiae, extracellular amino acids present within the growth environment induce the expression of several permeases. The induction requires a plasma membrane localized sensor complex, dubbed the SPS sensor (Forsberg and Ljungdahl, 2001a). Cells lacking any one of the three components of this sensor, the *SSY1*, *PTR3* and *SSY5* gene products, are unable to respond to amino acid stimuli (Barnes et al., 1998; Didion et al., 1998; Forsberg and Ljungdahl, 2001a; Iraqui et al., 1999; Jørgensen et al., 1998; Klasson et al., 1999). Ssy1p, the only integral membrane component, is a unique member of the AAP family that does not itself transport amino acids (Didion et al., 1998; Iraqui et al., 1999), instead it functions as a receptor for external amino acids, initiating signals that are transduced to the peripherally associated plasma membrane proteins Ptr3p and Ssy5p (Bernard and Andre, 2001; Forsberg and Ljungdahl, 2001a; Klasson et al., 1999). Stp1p and Stp2p are redundant transcription factors that bind to specific sequences within the promoters of SPS sensor regulated genes (de Boer et al., 2000; Nielsen et al., 2001). Stp1p and Stp2p are synthesized as latent cytoplasmic precursors (Andreasson and Ljungdahl, 2002). In response to extracellular amino acids, the SPS sensor induces the rapid endoproteolytic processing of Stp1p and Stp2p. The shorter forms of these transcription factors, lacking N-terminal inhibitory domains, are targeted to the nucleus where either can transactivate SPS sensor target AAP genes.

AAPs are a conserved family of integral membrane proteins that are localized to the plasma membrane (PM). As other PM proteins, AAPs are initially co-translationally inserted into the membrane of the endoplasmic reticulum (ER). Subsequent to membrane insertion, these proteins attain native conformations prior

to being transported from the ER to the Golgi apparatus via ER derived transport vesicles (reviewed by (Barlowe, 1998; Kaiser and Ferro-Novick, 1998; Springer et al., 1999). The members of the AAP family (Gilstring et al., 1999; Kuehn et al., 1996; Ljungdahl et al., 1992), including the SPS sensor component Ssy1p (Klasson et al., 1999), require Shr3p to exit the ER. In cells lacking *SHR3* amino acid permease family members accumulate in the ER membrane. Consequently, *shr3* null mutants are unable to respond to external amino acid cues and have a greatly reduced capacity to take up amino acids. Diploid strains carrying homozygous *shr3* null mutations undergo dimorphic transitions at enhanced frequency and exhibit excessive pseudohyphal growth (Gimeno et al., 1992). Shr3p is an integral membrane component of the ER with 4 membrane spanning segments and a hydrophilic cytoplasmically oriented carboxy-terminal domain. Shr3p physically associates with AAPs, but not with other polytopic membrane proteins, and facilitates the membrane association and assembly of the soluble vesicle coat forming components (COPII coatomer) (Gilstring et al., 1999). Shr3p functions as a packaging chaperone that directs the formation of vesicle buds around AAPs, and thereby ensures their inclusion into transport vesicles.

Our laboratory has contributed to the understanding of the molecular mechanisms governing amino acid uptake in the yeast S. cerevisiae. Based on this understanding we have assessed the importance of amino acid uptake in the growth and pathogenesis of the related yeast C. albicans. Due to the central role of Shr3p in amino acid uptake we have initially focused on its Candida homolog Csh3p. Our results indicate that Csh3p functions analogously in Candida as Shr3p does in Saccharomyces. Null mutations in *CSH3* reduce the capacity of amino acid uptake in a dose dependent manner. A homozygous *csh3Δ/csh3Δ* mutant is unable to switch morphologies on solid and in liquid media in response to inducing amino acids, whereas *CSH3/csh3Δ* heterozygous strains display normal amino acid induced morphological switching. Strikingly both *CSH3/csh3Δ* heterozygous and *csh3Δ/csh3Δ* homozygous strains are unable to efficiently mount virulent infections in

a mouse model. The haploinsufficiency phenotypes indicate that both *CSH3* alleles contribute to maintain high-capacity amino acid uptake in wild-type strains, and demonstrate that C. albicans cells depend on their ability to efficiently take up amino acids during growth in mammalian hosts.

Results

CSH3 Encodes a Structural and Functional Homolog of Shr3p

We sought to obtain C. albicans strains with severe and specific defects in amino acid uptake. The availability of such strains would subsequently enable us to assess the importance of the amino acids for growth and pathogenicity. In the first step towards this goal we cloned the C. albicans gene encoding the homolog of the S. cerevisiae packaging chaperone Shr3p (Table 1). Two different DNA fragments containing the *CSH3* (C. albicans *SHR3*) were amplified by PCR using genomic DNA isolated from strain SC5314 as template (Figure 1A). These fragments, 2.2 kb in length, correspond to the two alleles of *CSH3* (contained within contigs19-20179 and 19-10174 of the diploid genome assembly, respectively, Stanford Technology Center, Stanford University). The alleles can be distinguished by Southern analysis using ClaI restricted DNA. The *CSH3* ORFs differ at several nucleotides, however only one of the differences results in an amino acid change at position 213 (G or S) (Figure 1B). Csh3p is closely related to Shr3p (Figure 1B); these proteins share 36% sequence identity and 48% sequence similarity. The homology is dispersed throughout the length of the two proteins; significantly, there are no stretches of sequence identity longer than five amino acids. The positions and lengths of the four membrane spanning segments are well conserved.

We tested whether the heterologous expression of *CSH3* in S. cerevisiae could complement *shr3Δ* null mutations. In *shr3* mutant strains AAPs are retained in the ER. As a result of the reduced levels of AAPs in the PM, *shr3* mutants exhibit poor growth on media containing proline as the sole nitrogen source, are resistant to toxic

concentrations of histidine (Ljungdahl et al., 1992) and many toxic amino acid analogues (Martínez and Ljungdahl, 2000; Roberg et al., 1997). The growth characteristics of *shr3Δ6* null mutant strains (FGY145) carrying either a plasmid without insert (vector pRS316), or plasmids expressing *SHR3* (pPL210) or *CSH3* (pPM20) were examined (Figure 1C). All Ura$^+$ transformants grew equally well on ammonium based minimal media (SD). Cells carrying the vector control grew poorly on proline-based media (SPD) and were resistant to 30 mM histidine and the toxic amino acid analogues examined. Conversely, transformants carrying the *SHR3* plasmid grew well on SPD and were unable to grow in the presence of high histidine and all of the toxic amino acid analogs examined. Cells carrying the *CSH3*plasmid exhibited an indistinguishable pattern of growth as strains carrying the *SHR3* plasmid. These results demonstrate that the expression of *CSH3* in S. cerevisiae fully complements the *shr3* null mutant phenotypes, and indicate that Csh3p is able to function as a packaging chaperone that facilitates the exit of AAPs from the ER (Gilstring et al., 1999).

Construction of *csh3* Null Mutant Strains of C. albicans

Based on the findings that the heterologous expression of Csh3p complements *shr3* null mutations we posited that it would have an equally important role in the capacity of C. albicans cells to take up amino acids. To investigate this possibility we created a set of isogenic C. albicans strains (Table 2) lacking either one or both chromosomal copies of *CSH3* (see Experimental Procedures). The two *CSH3* alleles were sequentially disrupted in an Ura3$^-$ C. albicans strain (CAI4) using a two-step (pop-in pop-out) gene replacement strategy (Rothstein, 1991). The *CSH3/csh3Δ* heterozygous (PMRCA8) and homozygous *csh3Δ/csh3Δ* (PMRCA10) strains carry unmarked *csh3Δ3* null alleles that lack any exogenous sequences.

It is well recognized that in comparison to apparently isogenic Ura$^+$ strains, Ura$^-$ strains of C. albicans display a decreased capacity to undergo morphological transitions (Lay et al., 1998). Thus, the consensus is that experimental assessments

regarding metabolism and developmental events in C. albicans should be carried out in Ura$^+$ prototrophic strains. We therefore reintegrated the Ca*URA3* gene into one of its endogenous loci resulting in the following Ura$^+$ strains; *CSH3/CSH3* (+/+, PMRCA18), *CSH3/csh3Δ* (+/-, PMRCA19), and *csh3Δ/csh3Δ* (-/-, PMRCA12). Finally, to control for the possibility of transformation-induced mutations, the *CSH3* gene was reintroduced back into one of the endogenous loci resulting in strain *csh3Δ/csh3Δ::CSH3-URA3* (-/-::+, PMRCA13).

In the course of constructing this set of isogenic strains we noted that the commonly used Ura$^+$ control wild-type strains SC5314 (*URA3/URA3*) and CAF2-1 (*URA3/ura3Δ*), the parental strains used during the original construction of CAI4 (Fonzi and Irwin, 1993), exhibit obvious phenotypic differences compared to five independent transformants of CAI4 carrying the *URA3* gene correctly integrated into one of the endogenous loci. Specifically, SC5314 and CAF2-1 grew more robustly in liquid and on solid media, exhibited enhanced filamentous growth under all inducing conditions examined, and CAF2-1 was more virulent when systemically introduced into mice. Based on these observations, it is our opinion that SC5314 and CAF2-1 cannot be considered proper wild-type control strains when analyzing mutants derived from the CAI4 strain. Presumably, mutation(s) and/or genomic DNA rearrangements were inadvertently introduced during the construction of strain CAI4.

Csh3p Functions in C. albicans as Shr3p Functions in S. cerevisiae

I. csh3Δ3 null mutations pleiotropically affect amino acid uptake

To directly test the endogenous role of Csh3p we examined the uptake of amino acids and adenine in our C. albicans strains. Compared to the *CSH3/CSH3* (+/+) strain, the *csh3Δ/csh3Δ* (-/-) mutant exhibited a decreased capacity to transport each of the amino acids tested (Figure 2A). We could not detect measurable lysine or phenylalanine uptake into the *csh3Δ/csh3Δ* mutant, and the rates of histidine and proline uptake were greatly diminished. The *CSH3/csh3Δ* (+/-) heterozygous strain exhibited slight but significant decreases in the rates of lysine, phenylalanine and

proline uptake, suggesting that in wild-type strains both copies of *CSH3* are expressed. In contrast, the *csh3Δ/csh3Δ* mutant and the *CSH3/csh3Δ* heterozygous strain did not show decreased capacities to transport adenine, in fact, the rate of adenine uptake increased in both of these strains.

We tested the capacity of *csh3Δ* mutations to affect cell growth on different nitrogen sources. Equally dense suspensions of strains *CSH3/CSH3* (+/+), *CSH3/csh3Δ* (+/-), *csh3Δ/csh3Δ* (-/-) and *csh3Δ/csh3Δ::CSH3-URA3* (-/-::+) were prepared in H_2O and aliquots were spotted onto solid media containing a variety of nitrogen sources (Figure 2B and Figure 3). All strains grew equally well when non-amino acid nitrogen sources were used, i.e., ammonium, allantoin, and urea. As compared to the *CSH3/CSH3* wild-type, the *csh3Δ/csh3Δ* mutant grew less well on amino acid based media. The negative effect on growth was substantial when tryptophan and proline (Figure 2B), and citrulline (Figure 3A) were used as sole nitrogen sources. When glutamine and glycine were used a more subtle growth defect was noted (Figure 2B). The pleiotropic effects of *csh3Δ* mutations on amino acid uptake confirm that Csh3p has an important role in facilitating amino acid uptake in C. albicans cells.

II. Csh3p is a component of the ER

The intracellular location of Csh3p was determined by visualization of a functional C-terminal tagged Csh3p-GFP fusion protein (Experimental Procedures). PMRCA15 cells (*csh3Δ3/csh3Δ3::CSH3-GFP3*) were prepared for microscopic evaluation. The cells showed bright perinuclear rim staining that often extended in a filamentous manner into the cytoplasm (Figure 2C). The pattern of Csh3p fluorescence is similar to the immunostaining observed for Shr3p (Ljungdahl et al., 1992) and other ER proteins in S. cerevisiae (Deshaies and Schekman, 1990; Feldheim et al., 1992; Rose et al., 1989). The ER localization of Csh3p is consistent with Csh3p acting analogously to Shr3p as an AAP specific packaging chaperone (Gilstring et al., 1999; Kuehn et al., 1996; Ljungdahl et al., 1992; Martínez and Ljungdahl, 2000).

Heterozygous *CSH3/csh3Δ* Strains Exhibit Amino Acid Uptake Related Haploinsufficiencies

Several growth phenotypes associated with *csh3Δ* mutations were gene dosage dependent. When strains were grown on SUD media with urea as sole nitrogen source, the *CSH3/CSH3* (+/+) wild-type strain was sensitive to the toxic lysine analog 2-aminoethyl-L-cysteine (Figure 3A). In contrast, the *csh3Δ* (-/-) null mutant strain was resistant and exhibited robust growth. The heterozygous strains, either *CSH3/csh3Δ* (+/-) or *csh3Δ/csh3Δ::CSH3* (-/-::+), grew significantly better than the wild-type strain, however not as well as the homozygous *csh3Δ* null mutant strain. Haploinsufficiency was also observed when cells were grown on media containing citrulline as the sole nitrogen source and on YPD (Figure 3A). Compared to the wild-type strain, the heterozygous strains *CSH3/csh3Δ* (+/-) and *csh3Δ/csh3Δ::CSH3* (-/-::+) grew less well, but better than the homozygous *csh3Δ* (-/-) null mutant strain. It should be noted that the growth differences observed on YPD reflect rather subtle differences, the effects shown in Figure 3 are visible only after short incubations. However, the pattern of growth on YPD accurately reflects differences in the growth rates of the strains. The doubling times of our strains growing in YPD are as follows: *CSH3/CSH3* (+/+), 1.2 hours; *CSH3/csh3Δ* (+/-), 1.3 hours; *csh3Δ/csh3Δ* (-/-), 1.6 hours; and *csh3Δ/csh3Δ3::CSH3* (-/-::+), 1.3 hours. These results indicate that heterozygous strains exhibit amino acid uptake defects with a degree of severity intermediate to that of the *csh3Δ/csh3Δ* null mutant and wild-type strains.

A striking gene dosage effect of *csh3Δ* mutations was observed on lysine containing media (Figure 3B). The strains did not grow in the absence of lysine, whereas they grew weakly in the presence of 0.1 mM lysine. The ability of the homozygous *csh3Δ/csh3Δ* (-/-) to grow must reflect the uptake of lysine through residual permeases present in greatly reduced amounts, or by non-specific uptake systems. Consistent with this notion, the growth of the homozygous null mutant noticeably improved with increasing concentrations of lysine. In contrast, the growth

of the *CSH3/CSH3* (+/+) wild-type strain did not improve with increasing lysine concentrations, in fact concentrations > 1 mM had a growth inhibitory effect (Figure 3B, compare growth at 1 mM and 3 mM lysine). The impaired growth of the wild-type strain is likely due to the inability to restrict lysine uptake, suggesting that lysine exerts toxic affects when intracellular concentrations become too high. In contrast the heterozygous strains, *CSH3/csh3Δ* (+/-) and *csh3Δ/csh3Δ::CSH3* (-/-::+), exhibited robust growth on media containing lysine concentrations greater than 1 mM. Together these results indicate that C. albicans can use lysine as a nitrogen source, if its import into cells can be sufficiently slowed to mitigate its toxic effects. Due to haploinsufficiency, heterozygous strains have fortuitously balanced these opposing activities.

CSH3 is Required for Proper Amino Acid Induced Hyphal Development and Filamentous Growth

C. albicans is polymorphic fungus that can either grow as budding yeast or switch to filamentous forms (either hyphae or pseudohyphae) (Ernst, 2000a). In laboratory settings C. albicans cells undergo morphological transitions in response to a wide variety of environmental conditions. Conditions favoring morphological switching from yeast to filamentous growth include temperature shifts to ≥ 35 °C, maintaining culture conditions at ≥ pH 7, limiting nutrient levels, and the introduction of serum and certain amino acids into the culture media (Odds, 1988; Tripathi et al., 2002). In S. cerevisiae, dimorphic transitions resulting in pseudohyphal growth are greatly enhanced in diploid strains homozygous for *shr3* null mutations (Gimeno et al., 1992). Based on the high degree of functional conservation between Csh3p and Shr3p, we expected that C. albicans strains carrying *csh3Δ* mutations would exhibit altered capacities to undergo morphological transitions. We envisioned two possible outcomes. First, if nitrogen limitation resulting from the inability to take up amino acids generates an inducing signal, as is the case in S. cerevisiae, then strains lacking *CSH3* would exhibit an increased propensity to filament. Alternatively, if

amino acids directly or indirectly provided stimulatory signals, then C. albicans lacking *CSH3* would exhibit a decreased ability to filament.

I. growth on solid media

To test these possibilities we examined the ability of our strains to form hyphae on 10% serum agar and on solid Spider medium (Figure 4, panels A and B, respectively). Aliquots of cell suspensions with equal numbers of cells were spotted on these media, and hyphal growth was assessed by examining the edges of the giant colonies (i.e., colonies originating from multiple cells). The *csh3Δ/csh3Δ* (-/-) mutant exhibited clearly reduced hyphal growth on both of these media. In contrast, extensive and indistinguishable levels of hyphal growth was observed surrounding colonies of the *CSH3/CSH3* (+/+) wild-type and *CSH3/csh3Δ* (+/-) heterozygous and *csh3Δ/csh3Δ3::CSH3* (-/-::+) complemented strains. These observations show that the filamentation defects associated with *csh3Δ* mutations are recessive. Additionally, the morphology of giant colonies was affected by the *csh3Δ* mutation (Figure 4, panel C). The wild-type (+/+), heterozygous (+/-) and complemented (-/-::+) strains formed giant colonies that were extensively wrinkled whereas the colonies formed by the homozygous (-/-) strain were smooth. These results indicate that wild-type and heterozygous cells switch morphologies in response to the amino acids present in Spider medium and serum, and demonstrate that amino acids act as morphogens.

II. growth in liquid media

We examined the capacity of the strains to form hyphae in liquid Lee's medium. This synthetic medium, containing several amino acids, biotin, inorganic salts and glucose, was developed based on the aminopeptidase profile of C. albicans (Lee et al., 1975). A useful characteristic of Lee's medium is that C. albicans exhibits temperature dependent filamentous growth; at 25 °C cells grow in their yeast form, when shifted to 37 °C wild-type cells rapidly undergo morphological transitions and

form hyphae. Cells grown in Lee's medium at 25 °C were used to inoculate fresh Lee's medium and the incubation temperature was simultaneously raised to 37 °C, and the time course of hyphal development was monitored for a period of 24 hours (Figure 5A). Initially, the cells in all cultures exhibited an ovoid shape typical of diploid yeast cells. At the 1.5 hour time point, approximately 60% of the cells in each culture, regardless of their genotype, had small germ tube-like projections, indicating that the cells were responding to the temperature shift. At this point it was impossible to distinguish the different cultures. However clear differences were observed at the 5 and 24 hour time points. At these time points the majority of cells in cultures of *CSH3/CSH3* (+/+) wild-type, *CSH3/csh3Δ* (+/-) heterozygous and *csh3Δ/csh3Δ::CSH3* (-/-::+) complemented strains had long hyphal filaments. In contrast, even 24 hours post induction, the homozygous *csh3Δ/csh3Δ* (-/-) mutant cells did not exhibit detectable hyphal growth. However, the *csh3Δ/csh3Δ* mutant cells were slightly more elongated; apparently the germ tube-like extensions of the *csh3Δ/csh3Δ* mutants observed at the 1.5 hour time point ceased to elongate, and subsequently enlarged to form elongated daughter cells. These elongated cells resemble *cph1/cph1 efg1/efg1* double mutant cells that also lack the ability to form hyphae (Lo et al., 1997), and rod-like opaque cells (Miller and Johnson, 2002; Sonneborn et al., 1999). The differences in the extent of filamentous growth were clearly apparent when the 24-hour-old cultures were left stationary for 30 min (Figure 5B). Due to their inability to filament the majority of the *csh3Δ/csh3Δ* cells accumulated at the bottom of the tube (tube 3). In contrast, the extensive filamentation of cells in the cultures of wild-type (tube 1), heterozygous (tube 2) and complemented (tube4) strains formed a fluffy mycelial mass that remained suspended in the medium.

csh3/csh3 Mutants Undergo Morphological Transitions in Response to Non-Amino Acid Inducing Stimuli

Proline and the acylated amino sugar *N*-acetyl glucosamine (Glc-NAc) are known to be strong inducers of filamentation in C. albicans (Holmes and Shepherd, 1987). We examined whether these substances could independently induce morphological transitions in our wild-type (+/+) and *csh3Δ/csh3Δ* (-/-) cells. The strains were grown in YPD, washed and resuspended in buffer and buffer containing either 10 mM proline or 2.5 mM Glc-NAc. The cell suspensions were incubated at 37 °C and cells were microscopically examined after 12 hours (Figure 6A). In the absence of an inducing signal (buffer), neither the wild-type nor mutant cells exhibited hyphal growth characteristics. However, wild-type cells exposed to proline readily formed hyphae. In contrast, the *csh3Δ/csh3Δ* mutant cells exhibited a markedly reduced propensity to form hyphae, only a few cells with short germ tubes were observed. No differences between the strains were observed when hyphal growth was induced by Glc-NAc, both the wild-type and *csh3Δ* null mutant strains exhibited extensive hyphal development. Thus *csh3* null mutations do not affect the capacity of cells to sense and react to non-amino acid based stimuli. Consistently, *csh3Δ/csh3Δ* null mutants also formed hyphae when grown in medium 199 (pH 8) (Saporito-Irwin et al., 1995). In these media, the null mutant strain exhibited an indistinguishable pattern of growth as compared to the *CSH3/CSH3* wild-type strain (data not shown).

We followed the proline uptake characteristics of the uninduced (buffer) and proline induced cells during the first 5 hours of these experiments. At the start of the experiment the wild-type cells grown in YPD exhibited a rate of proline uptake (Figure 6B) that was approximately 3 fold higher than wild-type cells grown in SUD (compare with Figure 2A). As expected the *csh3Δ/csh3Δ* mutant cells had a low capacity to take up proline at all time points (Figure 6B). The uptake capacity of wild-type cells suspended in buffer (left panel) remained unchanged after one hour, but after 5 hours, the proline uptake capacity was down to the level of the mutant cells. In the presence of proline (right panel), the rate of uptake dropped significantly faster; after one hour the rate of proline uptake was as low as the *csh3Δ/csh3Δ* mutant. Presumably, the rapid loss of proline uptake in the wild-type cells was the result of

feedback inhibition. These results suggest that morphological transitions are initiated rapidly in response changing environmental conditions and are not the consequence of long term starvation effects. This conclusion is consistent with previous work demonstrating that a short pulse (< 1 hour) of proline is sufficient to induce germ tube formation (Dabrowa and Howard, 1981).

CSH3/csh3Δ Heterozygous and *csh3Δ/csh3Δ* Homozygous Strains Exhibit Diminished Virulence in Mice

The virulence of our strains was determined in a mouse model (see Experimental Procedures). Mice were intravenously injected with 0.15 ml of saline solution or saline solution containing 1 x 10^6 cells. The ability of mice to survive was monitored for a period of 30 days (Figure 7A). All mice injected with the *CSH3/CSH3* (+/+) wild-type cells died within 9 days. For purposes of comparison with other published virulence studies, the virulence of the commonly used wild-type control strain CAF2-1 was also tested. This strain mounted a more aggressive infection and after 5 days all ten of the injected mice had died (data not shown). In contrast, all cells with one intact or both alleles of *CSH3* deleted, i.e., *CSH3/csh3Δ* (+/-), *csh3Δ/csh3Δ* (-/-) and *csh3Δ/csh3Δ::CSH3-URA3* (-/-::+), exhibited an indistinguishable pattern of reduced virulence: 50% of the mice remained alive 16 days post-injection, and in all but one instance, one mouse from each group survived for the duration of the 30 day period. We isolated yeast cells from the kidneys of infected mice. The genotypes of the reisolated strains were determined by PCR using primers specific for the *CSH3* locus (Figure 7B). In all cases, the genotypes of the reisolated strains corresponded to that of the strain that was injected. The data, consistent with the haploinsufficiency observed with amino acid uptake (Figure 3), indicate that both copies of the *CSH3* are required for virulence.

Discussion

Our laboratory has contributed to the elucidation of the mechanisms regulating amino acid uptake in S. cerevisiae. Based on this understanding we have assessed the role of amino acid uptake in the growth and virulence of the related human pathogenic fungus C. albicans. Based on three observations we predicted that amino acid uptake would have a central role in both of these linked processes. First, amino acids have been implicated as inducers of morphological transitions that enable Candida to switch between yeast and filamentous (pseudo)hyphal growth forms (Dabrowa and Howard, 1981; Land et al., 1975; Prasad, 1987). Thus, amino acids may directly influence the molecular mechanisms that engage discrete developmental programs. Second, amino acids present within hosts are likely to constitute major sources of nitrogen required for growth, and therefore might have an intrinsic role in pathogenesis. Third, the gene products in S. cerevisiae required for the functional expression of amino acid permeases (Shr3p) and in the positive regulatory circuit controlling the transcriptional induction of amino acid permeases (SPS sensor pathway) have homologs in the C. albicans genome (Table I).

This study is based on the rationale that the role of amino acids can be assessed in a *csh3Δ* homozygous null mutant with a severe, but specific, defect in amino acid uptake. We have made several observations that strongly support the notion that Csh3p has the same critical role in positively facilitating amino acid uptake in C. albicans as its homolog Shr3p has in S. cerevisiae. The heterologous expression of Csh3p fully complements *shr3* null phenotypes, a finding that reflects a high degree of functional and structural conservation (Figure 1). The functional similarity of Shr3p and Csh3p suggested that C. albicans lacking Csh3p would have a diminished capacity to take up amino acids and to respond to amino acid based stimuli, an assumption that proved correct. Although the present study does not directly address the role of Csh3p in the transport of AAPs from the ER, the amino acid uptake defects (Figure 2A) and amino acid dependent growth characteristics of strains carrying *csh3* null alleles (Figure 2B and 3), and the ER localization of

Csh3p(Figure 2C) are consistent with Csh3p acting analogously to Shr3p as an AAP specific packaging chaperone (Gilstring et al., 1999; Kuehn et al., 1996; Ljungdahl et al., 1992; Martínez and Ljungdahl, 2000).

C. albicans cells undergo morphological transitions in response to a wide variety of environmental conditions. Although not definitely established (Gow et al., 2002), the ability to switch between various growth forms is thought to contribute to the virulence of C. albicans by promoting several stages of disease establishment and progression (Corner and Magee, 1997; Odds, 1994). Cells trapped in either the yeast (Lo et al., 1997; Rocha et al., 2001) or filamentous states (Braun and Johnson, 1997) are less virulent, findings that underscore the importance of understanding how morphological transitions are induced. Despite intensive efforts, the exact nature of morphogenetic signals and the underlying mechanisms enabling cells to distinguish or sense these signals are poorly understood. The construction of C. albicans strains carrying heterozygous and homozygous *csh3Δ* mutations allowed us to directly examine the role of amino acids in stimulating morphological transitions and virulence. Several significant findings were made.

Homozygous *csh3Δ/csh3Δ* mutants have a severely reduced capacity to switch morphologies in response to the presence of inducing amino acids in both liquid and on solid media (Figures 4-6). The diminished capacity of the *csh3Δ/csh3Δ* null mutants to form hyphae in liquid medium is indicative of a severe defect; mutations giving rise to minor defects often exhibit reduced filamentation on solid but not in liquid media (Ernst, 2000a). The discovery that these mutants switch morphologies normally in response to non-amino acid stimuli, e.g., Glc-NAc and basic pH, indicates that the lack of Csh3p results in the inability of cells to specifically detect an amino acid based stimulus. Clearly amino acids provide positive morphogenic signals in Candida. This is in striking contrast to the situation in S. cerevisiae where nitrogen limitation induces dimorphic transitions (Gimeno et al., 1992). In fact, homozygous *shr3Δ/shr3Δ* S. cerevisiae cells have an hyperfilamentation phenotype as a result of diminished amino acid uptake, suggesting that S. cerevisiae cells use their ability to

grow as pseudohyphae to forage for nutrients. The growth environments within mammalian hosts are likely to have high amino acid contents. Apparently C. albicans and S. cerevisiae use signals generated from amino acid assessments to achieve growth advantages that reflect differences in the selective pressures present in the environments where these two yeast have evolved.

Remarkably both *CSH3/csh3Δ* heterozygous and *csh3Δ/csh3Δ* homozygous strains lack the ability to efficiently mount virulent infections in a mouse model (Figure 7). The fact that *CSH3/csh3Δ* heterozygous cells are able to undergo morphological transitions as well as wild-type cells, but exhibit reduced virulence, indicates that the capacity to switch morphologies does not intrinsically enable Candida cells to be pathogenic. Furthermore these findings indicate that although C. albicans is a prototrophic organism it depends upon high-capacity amino acid uptake systems to efficiently infect mammalian hosts. The haploinsufficiency phenotypes indicate that both *CSH3* alleles contribute to maintain high-capacity amino acid uptake in wild-type strains. Based on these findings we conclude C. albicans cells rely on amino acids present within mammalian hosts to obtain sufficient nitrogen to grow. Consistent with this notion, amino acids are present at high levels, above millimolar concentrations, in human blood (Nicholson and Pesce, 2000). Additionally, C. albicans secrete proteinases that have been shown to be important in nutrient acquisition, these proteinases degrade host proteins providing cells with amino acids and peptides for growth (Hube and Naglik, 2001).

Based upon what is known regarding Shr3p function in S. cerevisiae, it is likely that mutations in *CSH3* negatively affect amino acid uptake by two distinct processes. Two predictions can be made. First, AAPs will be specifically retained in the ER of *csh3Δ* cells leading to reduced levels of permeases at the PM. The second prediction is based on the fact that C. albicans clearly possess identifiable homologs of the entire S. cerevisiae SPS sensing pathway (Table 1). The existence of these homologs suggests that C. albicans cells are able to actively assess extracellular amino acids. In *shr3Δ* null mutant strains the core component of the SPS sensor,

Ssy1p, is retained in the ER (Klasson et al., 1999). Thus we predict that the Candida Ssy1p homolog (orf6.6705) will be retained in the ER. Consequently, since SPS sensor initiated signals are required to depress the transcription of AAP genes (Andreasson and Ljungdahl, 2002), *csh3Δ* mutations are expected to exhibit decreased AAP gene expression.

The gene dosage effects observed for amino acid uptake, i.e., diminished capacity to use amino acids as sole nitrogen-sources, increased resistance to toxic amino acid analogs and decreased virulence, indicate that Csh3p is present at rate limiting amounts in heterozygous *CSH3/csh3Δ* strains. In contrast, the filamentation defects are fully recessive, heterozygous and wild-type strains exhibit an indistinguishable propensity to form filaments. Morphological transitions are likely to be induced in response to signals initiated from amino acid sensing mechanisms that are amplified by downstream signal transduction pathways. Thus, the switches that activate discrete developmental programs should be less sensitive to the reduced levels of Csh3p in heterozygous *csh3Δ* mutant strains. With respect to the recessive nature of the filamentous growth defect, our data is consistent with two possibilities. First, the presence of a single copy of *CSH3* provides cells with sufficient residual uptake capacity to enable amino acids to accumulate to levels that induce an internal nutrient sensor. Alternatively, the reduced levels of Csh3p are still sufficient to enable the Ssy1p homolog to reach the PM, permitting the assembly of functional SPS sensor complexes. Our current efforts are directed at explicitly testing and distinguishing these two possibilities. Additionally it would be of great interest to understand if and how morphogenic signals derived from amino acid assessments are integrated with morphogenic signals derived from other stimuli known to regulate switching, e.g., cAMP, pH-responsive modules, general control of amino acid biosynthesis (*GCN4*), mitogen-activated protein kinases, Czf1p, and Cph2p (Brown et al., 1999; Lane et al., 2001; Lengeler et al., 2000; Liu, 2001; Tripathi et al., 2002; Whiteway, 2000).

Effective drugs are available for the treatment of candidiasis, however, despite appropriate therapy, mortality resulting from systemic infections in immunocompromised patients approaches 30% (Gale et al., 1998; Wenzel and Pfaller, 1991). The increased prevalence of resistant strains indicates an urgent need for new antifungal agents that are free of side-effects (Ernst, 2000b; Sanglard, 2002). As Candida and humans are eukaryotic organisms sharing many pathways, specific targets need to be identified that are exclusively restricted to the microorganism. In this context, the pathways specifically regulating the functional expression of AAPs appears to provide promising candidate drug targets since homologs have not been found in mammals. Additionally, the reduced virulence exhibited by the heterozygous *csh3Δ* strains in a mouse model increases the attractiveness of Csh3p as a drug target. It has been described that mutations giving rise to haploinsufficiencies encode good candidates for drug targets, because in these strains small changes in protein levels result in a substantial decreases in fitness (Giaever et al., 1999). Finally, Shr3p is a well-conserved protein in fungi: homologs exist in *Schizosaccharomyces pombe*, Psh3p (Martínez and Ljungdahl, 2000), in *Aspergillus nidulans* (AI327826) and in *Botryotinia fuckeliana* (AL115875). Therefore drugs may be found to inhibit similar processes in other important fungal pathogens, including those infecting humans and agriculturally important species, both plant and animal.

Experimental Procedures

Cloning of *CSH3* and Plasmid Constructions

All plasmids and relevant oligonucleotides used in this study are listed in Table 1. A 2.2 kb DNA fragment, initiating 1 kb upstream and ending 0.5 kb downstream of the *CSH3* ORF, was PCR amplified using primers FI and RI and genomic DNA isolated from strain SC5314 as template (Figure 1A and Table 1). To facilitate subsequent

manipulations, the primers FI and RI were designed to introduce HindIII and BamHI restriction sites, respectively. The PCR product was ligated into the pCR2.1-TA-TOPO vector (Invitrogen), and the inserts of 4 independent plasmids were sequenced. One of the plasmids, pPM18, with sequences corresponding to those of one wild-type *CSH3* alleles (Stanford Technology Center Database) was subcloned into HindIII/BamHI digested pRS316 (Sikorski and Hieter, 1989) to create plasmid pPM20. A KpnI/SacI fragment of pPM20 was cloned into Bluescript KS (+), to yield plasmid pPM38. A deletion within the *CSH3* ORF from +121 (EcoRV) to +542 (PstI) was made by religation of EcoRV/PstI restricted and Klenow-blunted pPM38, creating plasmid pPM40. The KpnI/SacI cleaved DNA fragments containing the *CSH3* and *csh3Δ3* alleles from pPM38 and pPM40 were cloned into KpnI/SacI digested pSMS44 (Saporito-Irwin et al., 1995)(Gift from W.A. Fonzi), to yield pPM44 and pPM45, respectively. Plasmid pSMS44 contains a 2.1 kb EcoRV/XbaI DNA fragment containing *CaURA3* that has been cloned into SmaI/XbaI digested pUC18.

A PCR based strategy, using primers FI and R2, was used to create a SmaI site immediately preceding the STOP codon of the *CSH3* ORF (Figure 1A and Table 1). This fragment was cloned into pCR2.1-TA-TOPO vector creating pPM19. After determining the orientation of the insert, a HinDIII/BamHI fragment was excised and cloned into HinDIII/BamHI digested pRS316, resulting in plasmid pPM22. A SmaI/NaeI fragment containing the *yEGFP3* gene (from pUG35 (Cormack et al., 1997), a gift from J.H. Hegemann, http://www.mips.biochem.mpg.de) was ligated into SmaI digested pPM22. The resulting plasmid, pPM47, contains the *yEGFP3* gene ligated inframe with *CSH3 (CSH3-GFP)*. An XhoI/KpnI fragment containing the *CSH3-GFP* tagged allele was cloned into XhoI/KpnI restricted Bluescript KS(+), yielding plasmid pPM50. The KpnI/SacI fragment containing the*CSH3-GFP* from pPM50 was subcloned into KpnI/SacI restricted pSMS44 creating plasmid pPM57.

C. albicans Strains

The S. cerevisiae and C. albicans strains used in this study are listed in Table 1. C. albicans cells were transformed by the spheroplast method based on a protocol originally developed for the transformation of Pichia pastoris (De Backer et al., 2000). C. albicans *csh3Δ3* mutants were constructed from the *ura3/ura3* strain CAI4 (Fonzi and Irwin, 1993). The two *CSH3* alleles were disrupted sequentially by a two step gene replacement strategy. Plasmid pPM45, harboring the *csh3Δ3* deletion allele and the *CaURA3* gene, was linearized by restriction with PflMI. PflMI cuts in the 5' upstream region of *CSH3* (Figure 1A). The linear pPM45 was introduced into strain CAI4, and Ura$^+$ transformants carrying the deletion allele integrated by homologous recombination at the *CSH3* locus were selected. At this point the *csh3Δ* allele, the *URA3* gene, and the *CSH3* wild-type allele are tandem arranged at one of the chromosomal *CSH3* loci. Spontaneous Ura$^-$ papillants were selected by passaging strains on media containing 5-fluoroorotic acid. Papillants having lost the wild-type copy of *CSH3* and that retained the *csh3Δ* mutation were identified. One heterozygous mutant strain, designated PMRCA8, was subjected to a second round of directed integration/loop-out mutagenesis to obtain the homozygous *csh3Δ/csh3Δ* null mutant strain PMRCA10. The wild type CAI4, PMRCA8 and PMRCA10 were made Ura3$^+$ by integrating a 4.9 kb BglII/PstI DNA fragment containing the *CaURA3* gene into the endogenous *URA3* locus, generating strains PMRCA18, PMRCA19 and PMRCA12, respectively. The *CaURA3* gene was isolated from plasmid pLUBP (a gift from W. Fonzi). The *CSH3* gene was reintroduced back into its locus by transforming PMRCA10 with plasmid pPM44 linearized by PflMI to obtain strain PMRCA13. Strain PMRCA15 was obtained by transforming PMRCA10 with plasmid pPM57 linearized by restriction with BspEI, which cuts in the promoter region of the *CSH3-GFP* allele (Figure 1A). The Csh3p-GFP fusion protein was judged to be functional based on growth based assays; strain PMRCA15 grew well on media containing tryptophan as the sole nitrogen source and was sensitive to the toxic

lysine analog 2-aminoethyl-L-cysteine (data not shown). Both PCR and Southern analysis were used to confirm each of the steps of the strain construction.

Media and Culture Conditions

Standard yeast media and culture conditions for S. cerevisiae and C. albicans were as described (Sherman, 1991). Ura$^-$ strains of C. albicans were grown in media supplemented with uridine (25 μg/ml). SD medium supplemented with histidine was used to examine the growth of S. cerevisiae strains in the presence of toxic amino acid analogues (Sigma). The concentrations of analogs were as follows: 1 μg/ml L-canavanine (arginine analog (Grenson et al., 1966); 500 μg/ml L-azetidine-2-carboxylate (proline analog (Lasko and Brandriss, 1981); 100 μg/ml β-chloro- D,L-alanine (threonine analog (Arfin and Koziell, 1971); 400 μg/ml ρ-fluoro-D,L-phenylalanine (possible tyrosine or phenylalanine analog (Roberg et al., 1997); 300 μg/ml D,L-ethionine (methionine analog (Sorsoli et al., 1964); and 225 μg/ml 2-aminoethyl-L-cysteine (lysine analog (Grenson et al., 1966). SPD and SPD containing 30 mM histidine were prepared as described (Ljungdahl et al., 1992).

The ability of C. albicans strains to utilize different nitrogen sources was examined on succinate buffered YNB (without amino acids and ammonium sulfate) (pH 6) containing 2% glucose (buffered YNB). Media was made solid by addition of 2% of nitrogen free purified agar. The amino acids and other nitrogen sources were added to a final concentration of 3 mM unless otherwise indicated. The growth of C. albicans strains in the presence of toxic amino acid analogues was tested on buffered YNB containing 3 mM urea as the nitrogen source (SUD). The capacity of strains to filament was studied on 10% fetal bovine serum and on Spider medium made solid by addition of 2% agar (Liu et al., 1994). Filamentation was also examined in liquid cultures using either Lee's (Lee et al., 1975), YPD supplemented with 10% fetal bovine serum, media 199 at pH 8 (Saporito-Irwin et al., 1995), or 10 mM MES-Tris buffer (pH 6.4) supplemented with 10 mM proline or with 2.5 mM (Holmes and Shepherd, 1987). All filamentation assays were carried out at 37°C.

Amino Acid Uptake Assays

C. albicans strains were grown in SUD to an OD_{600} of 0.8, and amino acid and adenine uptake rates were assayed essentially as described (Martínez and Ljungdahl, 2000). Cells were harvested by centrifugation, washed once with buffer A (10 mM MES-Tris pH 6.4, 2mM MgCl2, 25 mM KCl), and resuspended at a density corresponding to 6.5 mg dry weight cells/ml in buffer A supplemented with 2% glucose. The rates of amino acid uptake were determined at a substrate concentration of 50 μM. ^{14}C-labeled amino acids, obtained from Amersham (300 mCi/ mmol) were diluted to obtain the desired concentration. The initial rates were determined; subsamples were removed at 10, 20, and 30 s; filtered and washed as described. The uptake rate for each amino acid was linear throughout the subsampling period.

Analysis of Virulence in a Mouse Model

BALB/c mice (male) weighing between 18 and 20g were used to test the virulence of PMRCA18, PMRCA19, PMRCA12 and PMRCA13 strains. Exponentially growing cells in YPD were washed twice and resuspended in saline solution to an OD_{600} of 1. The actual concentration was verified by plating on YPD to determine viable counts. The virulence of each strain was tested by injecting 0.15 ml of saline solution containing $1.2x10^6$ cells into the tail vein of a mouse. Ten mice were injected for each strain. A control group was injected with the same volume of sterile saline solution. Survival was scored daily over a period of a month. The kidneys from two mice from each group were removed and homogenized. An aliquot was plated on YPD and ten yeast colonies per mouse were analyzed by PCR to assess their genotype. The *CSH3* locus was PCR amplified with primers annealing at positions –938 (F2) and +773 (R3) relative to the start codon (Table 1). These studies were carried out in accordance to accepted guidelines governing the use of laboratory

animals and approved by the Karolinska Institute Ethics Committee that oversees experiments involving animals.

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Figures

Figure 1. *CSH3* Encodes a Functional Homolog of Shr3p.

(A) The chromosomal regions at the *CSH3* loci. The 2.2 kb genomic fragments (thick line) containing the two alleles of *CSH3* (open arrow) were amplified by PCR using primers F1 and R1. Relevant restriction endonuclease sites are indicated as follows: B, BspEI; C, ClaI; R, EcoRV; H, HpaI; Pf, PflMI; P, PstI and S, SpeI. The location of the allele specific ClaI site is within parenthesis. As a result of this polymorphism, two *CSH3* containing fragments, an 8.8 kb (within contig 19-10174) and a 4.1 kb (within contig 19-20179), are generated when genomic DNA is restricted with ClaI. The *csh3Δ3* allele lacks protein coding sequences between the EcoRV and PstI restriction endonuclease sites; this deletion results in a protein lacking amino acids 41-182.

(B) Shr3p and Csh3p sequences aligned using the GAP algorithm (GCG Wisconsin Sequence Analysis Package). A colon indicates identical amino acid residues and similar residues, in accordance to the BLOSUM62 amino acid substitution matrix, are marked with a period. Amino acids predicted to lie within membrane-spanning domains are represented by white letters within black boxes. The allele specific amino acids at position 213 of Csh3p are indicated with white letters within a gray box.

(C) The heterologous expression of *CSH3* in S. cerevisiae complements *shr3Δ* null mutant phenotypes. Cell suspensions of strain FGY145 (*shr3Δ6*) transformed either with plasmid pRS316 (vector), pPL210 (*SHR3*) or pPM20 (*CSH3*) were spotted on SD media with the indicated toxic amino acid analogs (see Experimental Procedures): canavanine (arginine analog); AzC (proline analog); chloro-ala (threonine analog); f-phenyl-ala (possible tyrosine or phenylalanine analog); ethionine (methionine analog); and 2-ae-cys (lysine analog). The suspensions were

also spotted on SPD and SPD containing 30 mM histidine. Culture plates were incubated at 30 °C for six days and photographed.

Figure 2. Csh3p Function and Intracellular Localization.

(A) C. albicans strains *CSH3/CSH3* (PMRCA18, black bars), *CSH3/csh3Δ* (PMRCA19, gray bars) and *csh3Δ/csh3Δ* (PMRCA12, white bars) were grown in YNB containing urea as the sole nitrogen source to an OD_{600} of 0.8, and the initial rates of amino acid and adenine uptake were determined as described in Experimental Procedures. The initial rates of uptake were determined in duplicate samples in a minimum of three independent experiments; the average rates are plotted. Error bars represent one standard deviation. A unit is defined as one nmol min^{-1}.

(B) The ability of C. albicans strains *CSH3/CSH3* (+/+, PMRCA18), *CSH3/csh3Δ* (+/-, PMRCA19), *csh3Δ/csh3Δ* (-/-, PMRCA12), and *csh3Δ/csh3Δ3::CSH3* (-/-::+, PMRCA13) to utilize different amino acids as nitrogen sources for growth was assessed. Aliquots of suspensions containing equal numbers of cells were spotted on succinate buffered YNB containing ammonium or 3 mM of the indicated amino acids. Culture plates were incubated at 30 °C for three days.

(C) Localization of functional GFP-tagged Csh3p. Strain PMRCA15 (*csh3Δ/csh3Δ3::CSH3-GFP*) was grown in YPD and cells were prepared for microscopic analysis. Left panel, cells viewed by Nomarski optics. Middle panel, yEGFP fluorescence. Right panel, DAPI staining.

Figure 3. Gene Dosage Dependent Phenotypes of *csh3Δ* Null Mutations.

(A) Aliquots of suspensions containing equal numbers of cells of C. albicans strains *CSH3/CSH3* (+/+, PMRCA18), *CSH3/csh3Δ* (+/-, PMRCA19), *csh3Δ/csh3Δ* (-/-, PMRCA12), and *csh3Δ/csh3Δ3::CSH3* (-/-::+, PMRCA13) were spotted onto SUD,

SUD containing 2-ae-cys (lysine analog), and buffered YNB containing 3 mM L-citrulline as sole nitrogen source. Culture plates were incubated at 30 °C for three days. Aliquots of cell suspension were also spotted onto YPD; note that the YPD plate was incubated at 30 °C for only 12 h.

(B) Aliquots of cell suspension as in (A) were spotted on buffered YNB media containing the indicated concentrations of L-lysine. Culture plates were incubated at 30 °C for three days.

Figure 4. Filamentous Growth Defect of *csh3Δ/csh3Δ* Null Mutants on Serum and Spider Media.

Aliquots of suspensions containing equal numbers of cells of C. albicans strains *CSH3/CSH3* (+/+, PMRCA18), *CSH3/csh3Δ* (+/-, PMRCA19), *csh3Δ/csh3Δ* (-/-, PMRCA12), and *csh3Δ/csh3Δ3::CSH3* (-/-::+, PMRCA13), were spotted on 10% serum-agar (A) and on solid Spider medium (B and C). Culture plates were incubated at 37 °C for five days, and the resulting giant colonies were photographed. Enlargements showing the edges of colonies are shown in (A and B), and whole colonies are shown in (C). Scale bars, 1 mm.

Figure 5. Filamentous Growth of Cells in Liquid Culture.

C. albicans strains *CSH3/CSH3* (+/+, PMRCA18, tube 1), *CSH3/csh3Δ* (+/-, PMRCA19, tube 2), *csh3Δ/ csh3Δ* (-/-, PMRCA12, tube 3), and *csh3Δ/csh3Δ3::CSH3* (-/-::+, PMRCA13, tube 4) were pregrown in Lee's medium at 25 °C (non-inducing temperature). (A) Time course of hyphae formation; at t = 0 equal numbers of cells were inoculated into fresh Lee's medium and cultures were placed at 37 °C to induce filamentous growth. At the times indicated, cultures were microscopically examined. Scale bar, 10 μm. (B) Photographs of the culture tubes after an overnight incubation at 37 °C.

Figure 6. *CSH3* is Required for the Induction of Filamentous Growth by Amino Acids.

(A) Strains *CSH3/CSH3* (+/+, PMRCA18) and *csh3Δ/csh3Δ* (-/-, PMRCA12) were grown in YPD and cells were resuspended to an OD_{600} of 1 in buffer (10 mM MES, pH 6.4) or in buffer containing either 10 mM proline or 2.5 mM N-acetyl-glucosamine and incubated at 37 °C for 12 hours, after which cell suspensions were microscopically examined. Scale bar, 10 μm. (B) The proline uptake capacity of the cells suspended in buffer (right panel) and buffer containing 10 mM proline (left panel) was monitored during the first 5 hours of incubation. Initial uptake rates were determined in triplicate at the times indicated. The average values are plotted, and the error bars represent one standard deviation. A unit is defined as one nmol min^{-1}.

Figure 7. Virulence Assay

(A) Male BALB/c mice were injected with 0.15 ml of saline solution (open triangles) or with saline solution containing either of the following C. albicans strains; *CSH3/CSH3* (1.2×10^6 CFU of PMRCA18, +/+ closed squares), *CSH3/csh3Δ* (1.2×10^6 CFU of PMRCA19, +/- open squares), *csh3Δ/csh3Δ* (1.2×10^6 CFU and 1.3×10^6 CFU of PMRCA12, -/- closed and open circles), or *csh3Δ/csh3Δ::CSH3* (0.9×10^6 CFU of PMRCA13, -/-::+ closed triangles). Ten mice were used for each experiment.
(B) Yeast cells were isolated from the kidneys of infected mice, and DNA was prepared from 10 independent isolates from each kidney. PCR amplification, using primers annealing at positions –938 (F2) and +773 (R3) relative to the start codon of the *CSH3* ORF, was used to assess the genotype of each of the yeast isolates. The EtBr stained reaction products were separated by electrophoresis through a 0.7% agarose gel. The first lane of each group, c, corresponds to DNA from the laboratory strain injected into mice, and lanes 1 – 3 correspond to DNA obtained from three independent yeast strains reisolated from infected mice.

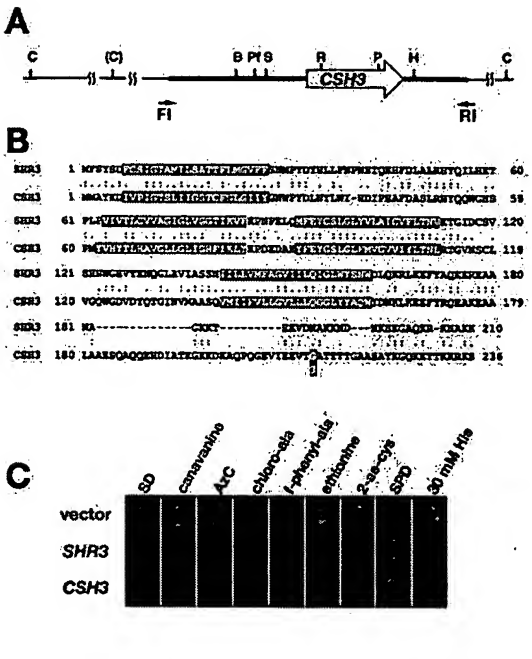

Figure 1

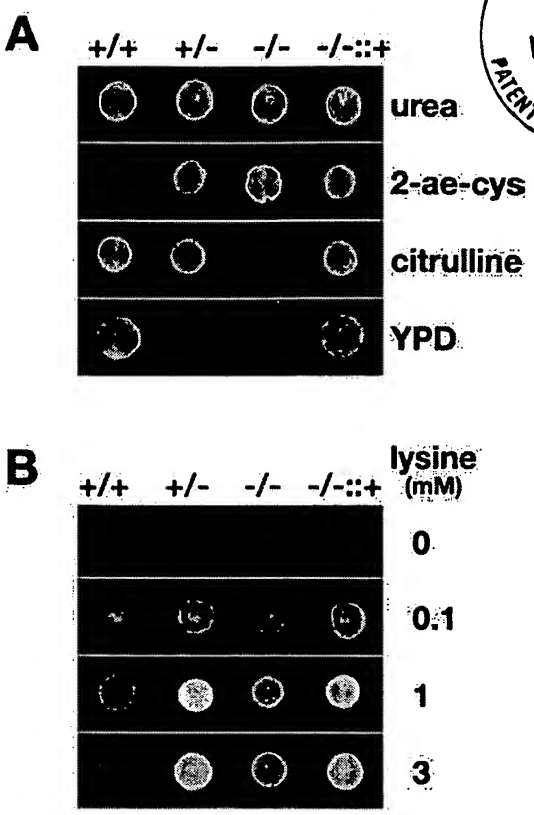

Figure 3

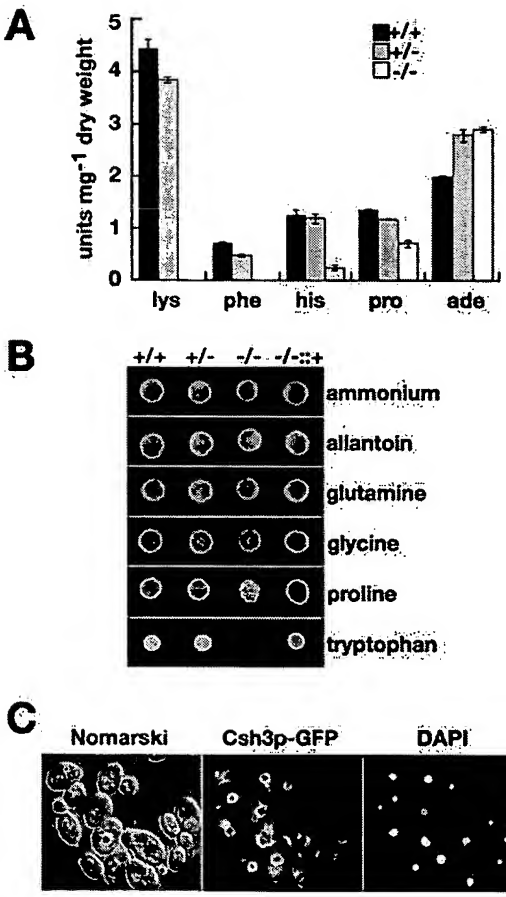

Figure 2

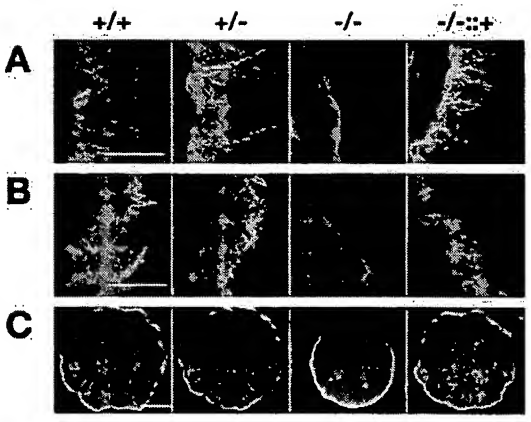

Figure 4

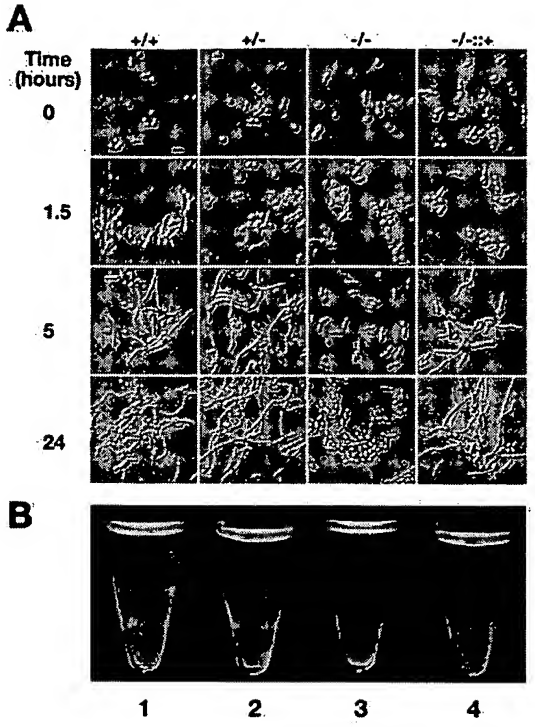

Figure 5

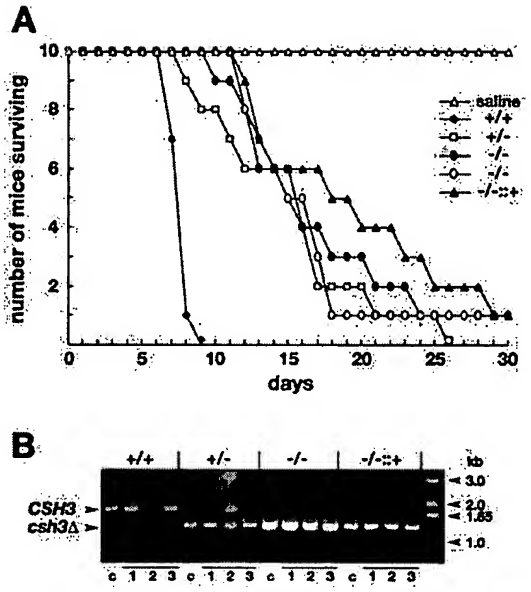

Figure 7

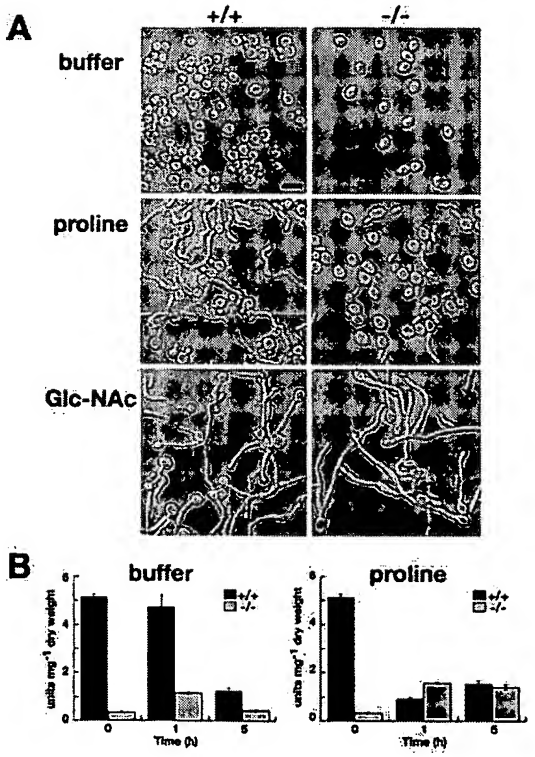

Figure 6

Table 1. Comparison of amino acid permeases and proteins involved in their functional expression in S. cerevisiae and C. albicans

S. cerevisiae	C.albicans	BLASTP probability P(N)	Gap results (%)* Similarity	Identity
Amino acid permeases				
Can1p	orf6.2072p	$2.4x10^{-146}$	69.7	61.5
Gap1p	orf6.7739p	$1.9x10^{-167}$	69.1	60.4
Can1p	orf6.2565p	$3.1x10^{-167}$	67.6	59.0
Can1p	orf6.2500p	$1.1x10^{-157}$	66.8	58.5
Agp3p	orf6.3068p	$1.3x10^{-72}$	66.7	56.5
Gap1p	orf6.4609p	$8.6x10^{-149}$	63.7	54.1
Gap1p	orf6.4378p	$9.5x10^{-127}$	63.2	52.7
Gap1p	orf6.2231p	$8.5x10^{-129}$	62.7	52.2
Gap1p	orf6.713p	$3.5x10^{-120}$	60.1	51.6
Gap1p	orf6.6768p	$1.1x10^{-130}$	59.8	50.2
Gap1p	orf6.7269p	$2.0x10^{-133}$	58.4	48.6
Gnp1p	orf6.2846p	$1.6x10^{-123}$	58.2	46.7
Gnp1p	orf6.8914p	$3.6x10^{-130}$	56.5	47.3
Gap1p	orf6.4583p	$2.0x10^{-110}$	56.3	44.3
Agp2p	orf6.5457p	$1.1x10^{-96}$	53.0	42.6
Tat1p	orf6.6050p	$1.3x10^{-115}$	52.2	40.8
Dip5p	orf6.8497p	$2.2x10^{-72}$	50.1	38.2
Dip5p	orf6.3623p	$2.7x10^{-81}$	48.5	36.6
Lyp1p	orf6.3531p	$7.2x10^{-58}$	47.5	36.0
Lyp1p	orf6.5571p	$2.5x10^{-71}$	45.6	35.9
Put4p	orf6.6671p	$2.5x10^{-78}$	45.0	36.3
SPS sensing pathway				
Ssy1p	orf6.6705p	$5.4x10^{-128}$	49.6	38.5
Ssy5p	orf6.7832p	$1.1x10^{-65}$	46.4	38.1
Ptr3p	orf6.6681p	$2x10^{-53}$	45.7	35.3
Stp2p	orf6.9120p	$2x10^{-30}$	41.9	33.2
Stp1p	orf6.2844p	$1.0x10^{-36}$	36.6	28.1
AAP specific packaging chaperone				
Shr3p	orf6.1787p	$2.8x10^{-35}$	48.3	35.9

*Calculated using the BLOSUM62 amino acid substitution matrix

TABLE 2. Yeast strains, plasmids and oligonucleotides

Strain	Genotype	Reference or source
S. cerevisiae		
FGY145	*MATa his4Δ29 ura3-52 shr3Δ6 GAL⁺*	Gilstring *et al.*, 1999
C. albicans		
SC5314	prototrophic wild-type	Fonzi and Irwin, 1993
CAI4	*ura3Δ::imm434/ura3Δ::imm434*	Fonzi and Irwin, 1993
PMRCA8	*ura3Δ::imm434/ura3Δ::imm434 CSH3/csh3Δ3*	This work
PMRCA10	*ura3Δ::imm434/ura3Δ::imm434 csh3Δ3/csh3Δ3*	This work
PMRCA12	*ura3Δ::imm434/URA3 csh3Δ3/csh3Δ3*	This work
PMRCA13	*ura3Δ::imm434/ura3Δ::imm434 csh3Δ3/csh3Δ3::URA3 CSH3*	This work
PMRCA15	*ura3Δ::imm434/ura3Δ::imm434 csh3Δ3/csh3Δ3::URA3 CSH3-GFP*	This work
PMRCA18	*ura3Δ::imm434/URA3*	This work
PMRCA19	*ura3Δ::imm434/URA3 CSH3/csh3Δ3*	This work
Plasmids	**Description**	
pPL210	1.4 kb AccI fragment containing *SHR3* in pRS316	Ljungdahl et al., 1992
pPM20	2.2 kb HinDIII/BamHI fragment containing *CSH3* in pRS316	This work
pPM44	2.2 kb KpnI/SacI fragment containing *CSH3* in pSMS44	This work
pPM45	1.8 kb KpnI/SacI fragment containing *csh3Δ3* in pSMS44	This work
pPM57	2.7 kb KpnI/SacI fragment containing *CSH3-GFP* in pSMS44	This work
Oligonucleotide	**Sequence**	
F1 (HinDIII)	CGAAAATGAGCACAAGCTTCAGCCCACGGCGAA	
F2	GGAGAATGTGGACCATATTCTGCA	
R1 (BamHI)	GTGAGTCTGCTGCGGATCCATACACCAAA	
R2 (SmaI)	CGACGAGTTTACCCGGGCGATTTTCTTT	
R3	TGAGCCTTATTGGTTAACCAG	